Annu. Rev. Plant Physiol. Plant Mol. Biol. 1998. 49:223–47

DNA METHYLATION IN PLANTS

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KEY WORDS: DNA methyltransferase, epigenetics, defense, gene silencing, development

ABSTRACT

Methylation of cytosine residues in DNA provides a mechanism of gene control. There are two classes of methyltransferase in Arabidopsis; one has a carboxy-terminal methyltransferase domain fused to an amino-terminal regulatory domain and is similar to mammalian methyltransferases. The second class apparently lacks an amino-terminal domain and is less well conserved. Methylcytosine can occur at any cytosine residue, but it is likely that clonal transmission of methylation patterns only occurs for cytosines in strand-symmetrical sequences CpG and CpNpG. In plants, as in mammals, DNA methylation has dual roles in defense against invading DNA and transposable elements and in gene regulation. Although originally reported as having no phenotypic consequence, reduced DNA methylation disrupts normal plant development.

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1040-2519/98/0601-0223$08.00

INTRODUCTION

The information content of the primary DNA sequence can be enhanced by addition of a methyl group to the ring structure of cytosine or adenine residues. Chemical modification of DNA affects protein-DNA interactions; in prokaryotes, modification of DNA by methyltransferases prevents cleavage by the cognate restriction endonucleases (reviewed in 102). In higher eukaryotes, cytosine methylation can inhibit binding of regulatory proteins (reviewed in 39), and methylation of promoter and coding sequences of genes can repress transcription, both in vitro and in vivo (reviewed in 64). Methylation of DNA has been implicated in the timing of DNA replication; in determination of chromatin structure; in increasing mutation frequency; as a causal agent for some human diseases; and as a basis for epigenetic phenomena (reviewed in 64). It has been suggested that DNA methylation in mammals reduces the background of nonspecific transcripts from complex genomes (25) and is a mechanism to maintain the many transposable elements present in the mammalian genome in a quiescent state (16, 18, 38).

Although the evidence that DNA methylation plays a role in the developmental regulation of gene expression is largely correlative, targeted mutations of the mouse DNA methyltransferase have demonstrated that DNA methylation is essential for normal mammalian development. Mouse embryos homozygous for a methyltransferase knockout mutation that reduces methylation to about 30% of normal abort spontaneously during gestation (81). The two parental genomes are not functionally equivalent in mammalian development, and a number of genes, known as imprinted genes, are differentially expressed depending on their parental origin. In methyltransferase-knockout embryos, imprinted genes from both maternal and paternal genomes are expressed equally (80), confirming that DNA methylation is essential for the maintenance of genomic imprinting. X chromosome inactivation is also perturbed in embryos with reduced DNA methylation because expression of *Xist*, a gene important for X chromosome inactivation, is aberrant (9, 111). In embryos with reduced levels of methylation, *Xist* is transcribed from each X chromosome, resulting in inappropriate X inactivation, which may contribute to embryo lethality (104).

Plants that have substantially reduced levels of DNA methylation also display a number of phenotypic abnormalities (46, 67, 119). In this review, we discuss the distribution of methylcytosine in plant genomes and the regulation of DNA

methylation. We consider the role of DNA methylation in regulating gene expression, as a mechanism controlling transposable elements, and as a defense against foreign DNA.

DISTRIBUTION OF METHYLCYTOSINE IN PLANT GENOMES

Early papers reported that methylation in plant DNA occurs predominantly in cytosines of symmetrical sequences such as CpG and CpNpG (54, 91a). This distribution of methylcytosine was determined using methylation-sensitive restriction enzymes and nearest-neighbor analyses modified to allow determination of trinucleotide sequences. While these conclusions were to some extent dictated by the choice of restriction enzymes, nearest neighbor analyses indicated that methylation occurred more frequently in CpApG and CpTpG than in CpApT (54). The strand symmetry of CpG and CpNpG motifs provides an obvious mechanism for transmitting methylation patterns through cycles of cell division (Figure 1).

More recently, genomic sequencing (49) has shown that methylation of cytosines in nonsymmetrical sites often occurs (97, 103, 141). In 182 bp of the promoter of a silenced chimeric 35S*A1* transgene in Petunia, 27 cytosine residues located in CpG or CpNpG (100%) and 40 cytosines in asymmetric sequences (69%) were either completely or partially methylated (97). Methylation in the promoter region of the tobacco T85 gene was observed in pollen DNA while the corresponding region was unmethylated in leaf DNA. In the region examined, 10 out of 49 cytosines were highly methylated but only three methylated cytosines were located in symmetric sites, suggesting that methylation at asymmetric sites may also be common in endogenous genes (103).

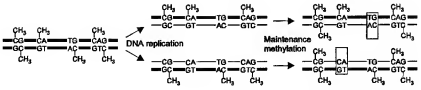

Figure 1 Transmission of methylation patterns through a cycle of DNA replication. Patterns of methylation based on cytosines located in symmetric sequences (CpG and CpNpG) are transmitted to both daughter strands following replication by the action of a methyltransferase, which preferentially methylates hemimethylated DNA at the replication fork, a process known as maintenance methylation. Methylation of cytosines in nonsymmetric sequences (CpXpX where X is any base other than G) is not transmitted to the newly synthesized daughter strand. Parental strand, *thick line*; newly synthesized strand, *thin line*. Loss of methylation at nonsymmetric sites on the daughter molecules is indicated by *box* surrounding these residues.

Methylation of symmetrically located cytosines may act as a nucleation center for the spread of methylation to adjacent nonsymmetric cytosines. The methylation pattern of individual DNA molecules was examined for the 35S*A1* transgene (97). Methylation of cytosine residues in CpG and CpNpG occurred at 100% of sites in almost every molecule. In contrast, many nonsymmetric sites were methylated at low frequency, and the pattern of methylation at these sites was variable between molecules. Methylation at these sites was probably not transmitted through cycles of DNA replication (Figure 1).

In plants, DNA methylation is mainly restricted to the nuclear genome, suggesting that the smaller chloroplast and mitochondrial genomes do not require this additional level of gene control (reviewed in 43). Methylcytosine is not randomly distributed throughout the nuclear genome but is concentrated in repeated sequences. Repetitive DNA consists of long tandem arrays that are normally clustered around the centromere, at telomeres, or in the nucleolar organizer region and of middle to highly repetitive sequences made up of retroelements and derivatives. At least 50% of the maize genome is composed of different families of retroelements (122); in bean, there are more than one million copies of one *copia*-like retroelement that makes up about 25% of the genome (47). It is likely that most methylcytosine in plant genomes, as in mammalian genomes (151), is located within retroelements that are heavily methylated (11, 13, 89). Modification and condensation into heterochromatin render retroelements transcriptionally and recombinationally inactive (13).

Approximately 80% of cytosines in CpG dinucleotides in plant genomes are modified (54), but despite this plants also contain regions of unmethylated DNA with closely spaced, unmethylated *Hpa*II restriction enzyme sites (CCGG). *Hpa*II digestion released a prominent low-molecular-weight DNA fraction with fragments ranging in size from ~25 bp to ~250 bp (3) that resemble the *Hpa*II tiny fragments characteristic of CpG islands in vertebrate genomes (35). Islands are typically unmethylated in a wide range of tissues whether or not the associated gene is transcribed (24). Unmethylated CpG islands have been identified in the maize *A1* (dihydroflavonol 4-reductase), *Adh1* (alcohol dehydrogenase), and *Sh1* (sucrose synthase) genes (3, 101).

METHYLATION OF DNA

DNA methyltransferase catalyzes transfer of a methyl group from *S*-adenosylmethionine (*S*-adomet) to the pyrimidine ring of cytosine residues (22, 75) in newly replicated DNA (23) (maintenance methylation, Figure 1). Changes in methylation patterns occur by de novo methylation or by passive demethylation through failure of maintenance methylation. Active demethylation has been reported in chicken and mouse (63, 144) but not yet in plants.

Plant DNA Methyltransferases

Prokaryote cytosine 5 (5mC) methyltransferases belong to a single class of enzyme consisting of 10 conserved protein motifs (I–X), arranged sequentially and separated by nonconserved sequences of variable length (76, 113, 130). Motifs I and X form the binding site for the cofactor *S*-adomet (32, 60). The cysteine residue of a completely conserved proline-cysteine doublet in motif IV forms the active site involved in methyl transfer (31, 98, 147, 148). The variable region between motifs VIII and IX—the target recognition domain (5, 72, 135, 147)—directs the enzyme to the recognition sequence flanking the target cytosine.

Eukaryote cytosine methyltransferases (2, 14, 19, 44, 70, 132, 150, 152) also have a conserved structure including a carboxy-terminal methyltransferase domain containing 8 of the 10 prokaryote motifs. In most eukaryote enzymes, this domain is fused to a large regulatory amino-terminal domain (Figure 2) which in the mouse enzyme, Dnmt1, targets the protein to the replication fork in S phase nuclei (78) and causes preferential methylation of hemimethylated templates (17). Mouse ES cells, homozygous for a null mutation of the Dnmt1 methyltransferase, contain low but stable amounts of methylcytosine and can methylate incoming retroviral DNA, demonstrating that there is a second methyltransferase in mouse (77). A second putative methyltransferase gene, Dnmt2, which appears to lack an amino-terminal domain, has been cloned from both mouse and human (JA Yoder & TH Bestor, personal communication).

There are two classes of genes encoding putative DNA methyltransferases in Arabidopsis. One class, represented by *METI* and *METII*, encodes proteins that are similar to the mouse Dnmt1 methyltransferase (Figure 2). Homology between *METI* and *METII* is higher in the methyltransferase domain

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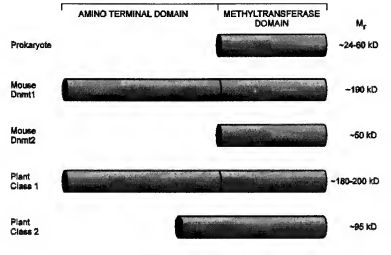
Figure 2 Comparison of methyltransferase proteins from prokaryote, mammalian, and plant sources. (*a*) Schematic representation of the methyltransferase structure showing the location of the methyltransferase and amino terminal domains and the approximate molecular weights of the different proteins. Plant class 1 methyltransferases include METI and METII from Arabidopsis, the two carrot methyltransferases (14), and the pea methyltransferase (S Pradhan & R Adams, personal communication). Arabidopsis METIII is the sole representative of a Plant class 2 methyltransferase (S Henikoff & L Comai, personal communication). (*b*) Amino acid identity between the different methyltransferases. Comparisons for the methyltransferase domain shown in the *lower half* of the figure include amino acids from the beginning of conserved motif I (113) to the carboxy-terminus. The *upper half* of the figure shows the comparisons for the amino-terminal domain spanning the amino-terminus to the beginning of the lysine-glycine repeat separating the two domains (19, 44). Sequence data for this figure were taken from the following references: 14, 44, 150, 152; RK Genger, unpublished observation; S Henikoff & L Comai, personal communication; JA Yoder & TH Bestor, personal communication.

than in the amino-terminal domain; these proteins are more divergent than the human and mouse Dnmt1 enzymes (44; RK Genger, unpublished observation). A third putative methyltransferase gene (*METIII*), identified in a database search of genomic sequence (H Goodman, unpublished observation), does not cross hybridize with *METI* or *METII* and belongs to the second class of methyltransferases (Figure 2) (EJ Finnegan, unpublished observation). The



(b)

	Mouse Dnmt1	Mouse Dnmt2	Human DNMT1	Arabid METI	Arabid. METII	Arabid METIII	Carrot MET5	Carrot MET21
Mouse Dnmt1		-	72	22	22	-	19	21
Mouse Dnmt2	22		-	-	-	-	-	-
Human DNMT1	83	21		22	22	-	22	23
Arabid. MET1	50	23	53		68	-	54	55
Arabid. METII	47	24	50	79		-	54	55
Arabid. METIII	24	25	34	34	34		-	-
Carrot MET5	51	26	53	76	71	37		90
Carrot MET21	51	27	53	77	74	35	92	

Amino terminal domain

Methyltransferase domain

predicted METIII protein lacks an amino-terminal domain and has a chromodomain, a short motif found in chromatin-associated proteins, Heterochromatin 1 (Hp1) and Polycomb (Pc) from Drosophila, inserted between motifs II and IV (S Henikoff & L Comai, personal communication).

Two very closely related DNA methyltransferase genes encoding class 1 methyltransferases (Figure 2) have been cloned from carrot (14). The two carrot methyltransferases, which are probably the products of a recent duplication, have higher homology to Arabidopsis METI than METII in the methyltransferase domain, suggesting that both are METI homologues (Figure 2).

A single methyltransferase cDNA encoding a protein similar to METI has been cloned from pea; in vitro assays with the expressed protein ($M_r \sim 180$ kDa) suggest that it has the capacity to methylate both CpG and CpA/TpG (S Pradhan & R Adams, personal communication). This conflicts with earlier studies reporting partial purification of two methyltransferases from pea, one of which preferentially methylates CpG ($M_r \sim 150$ kDa) and the other CpA/TpG ($M_r \sim 110$ kDa) (114). Proteolytic processing to yield smaller proteins in vivo has been invoked to reconcile the observed differences in protein size. Smaller proteins ($M_r \sim 50$–85 kDa) with methyltransferase activity have also been partially purified from wheat and rice (50, 133, 136); these may be the products of proteolytic cleavage.

Crystallization of a ternary covalent complex between a synthetic oligonucleotide template, purified *Hha*I methyltransferase, and *S*-adenosyl homocysteine (the product of methyltransfer from *S*-adomet) showed that the DNA is bound in a cleft between the catalytic and target recognition domains of the enzyme (32, 71). Methyltransfer involves extrusion of the target cytosine from the DNA helix, leading to distortion of the DNA backbone. A second prokaryote methyltransferase, *Hae*III, also gains access to target cytosines by their extrusion from the helix (117). Modeling the 3D structure of the METI methyltransferase domain, based on the *Hha*I methyltransferase crystal structure (32, 108, 109, 110), shows that the tertiary structure is conserved between a bacterial and a plant methyltransferase (RK Genger, unpublished observation). It is likely that these enzymes use similar mechanisms to access their target cytosine.

The identification of multiple DNA methyltransferases in plants raises the question of whether the proteins recognize and methylate cytosines in different sequence contexts, whether the different enzymes catalyze maintenance or de novo methylation, or whether they are active in different tissues or stages of development. There is evidence supporting the notion that plant methyltransferases may differ in target specificity. Two purified pea methyltransferases, which may originate from a single gene, differ in target specificity (114). In tobacco, methylation of cytosines in CpG dinucleotides and in CpNpG sequences showed differential sensitivity to methylation inhibitors 5-azacytidine (5-azaC)

and ethionine, suggesting that different enzymes may catalyze methylation of CpG and CpNpG sites, respectively (73). A *METI* antisense preferentially reduced methylation of cytosines in CpG and CpCpG sequences in transgenic Arabidopsis (46).

Most of the known plant methyltransferase transcripts are expressed ubiquitously in vegetative and reproductive tissues (14, 119; RK Genger, unpublished observation) but generally show higher expression in meristematic cells (14, 119). However, *METI* transcripts are at least 10,000-fold more abundant than those of *METII* (RK Genger, unpublished observation). *METIII* was expressed at low levels in both vegetative and reproductive tissues (EJ Finnegan, unpublished observation); expression was higher in floral tissue (S Henikoff & L Comai, personal communication), which is perhaps indicative of a role during gametogenesis. In some ecotypes of Arabidopsis, the protein is truncated by insertion of a retroelement upstream of some of the conserved motifs required for methyltransfer (S Henikoff & L Comai, personal communication). This shows that METIII is not essential for apparently normal development.

DNA Methylation Requires Proteins Other Than Methyltransferases

The first hint that proteins other than methyltransferases may be required for DNA methylation came from the identification of Arabidopsis mutants (*ddm*) at four loci that have decreased DNA methylation of the centromeric repeat (137). Mutations at *ddm1* and *ddm2* mutations are recessive, implying loss of function, but the remaining two mutations, *ddm0* and *ddmB*, are dominant or semidominant (EJ Richards, personal communication). *DDM2* maps to the previously described *METI* locus, but the other loci do not cosegregate with known methyltransferase genes (EJ Richards & J Jeddeloh, personal communication). *DDM1* does not encode a methyltransferase because *ddm1* mutants have normal levels of both CpG and CpNpG methyltransferase activity in vitro. The intracellular pools of *S*-adomet are also normal. DDM1 is only required for methylation in vivo and may have a role in chromatin structure, or in the interaction between methyltransferases and DNA (67).

Demethylation of both centromeric and ribosomal repeat sequence DNA was observed in M2 homozygous *ddm1* mutants; after repeated rounds of self-pollination, single copy sequences became demethylated in homozygous progeny (67). Demethylation of cytosines in both CpG and CpNpG sequences was observed in *ddm1* homozygotes (137); the effect on methylcytosine in nonsymmetric sequences has not been measured.

Regulation of De Novo Methylation

There are many unanswered questions about the regulation of DNA methylation; for example, what regulates de novo methylation of some but not all

cytosines in CpG and CpNpG motifs? Does methylation at asymmetric cytosines depend on methylation at adjacent symmetric sites? Is methylation regulated by chromatin structure or by other DNA-binding proteins that compete with the methyltransferase for access to DNA?

Transgenes (and transposable elements) that have been inactivated and methylated de novo show a high density of methylcytosine, in CpG, in CpNpG, and in asymmetric sequences. In some cases, methylation does not extend beyond the ends of the foreign DNA or transposon (95, 141); in other examples, methylation extends, at lower density, into the flanking plant DNA (86, 123).

De novo methylation of endogenous genes has been studied in plants in which DNA methylation has been perturbed by either mutation, antisense technology, or treatment with 5-azaC. Homozygous *ddm1* mutant plants, which had about 30% of normal methylation, were outcrossed to the wild-type progenitor. The level of methylation in progeny was intermediate between the mutant and wild-type parent, suggesting that even though the *ddm1* mutation is recessive, sequences that were demethylated in the genome of the mutant parent remained hypomethylated in the progeny (137). Repeated backcrossing to a wild-type parent showed that the level of methylation in each generation was intermediate between the parental lines, consistent with dilution of the hypomethylated genome rather than replacement of methylcytosine by de novo methylation (137).

Arabidopsis plants carrying a *METI* antisense transgene have reduced levels of DNA methylation (46, 119). When *METI* antisense plants, hemizygous for the transgene, were outcrossed to wild-type plants the hypomethylation phenotype was transmitted to progeny that did not inherit the antisense transgene (119). In a different study, gradual recovery of methylation levels was observed in progeny of selfed hemizygous methyltransferase antisense plants that did not inherit the transgene (antisense-null) (46). Methylation in first generation antisense-null plants was significantly below normal but was higher than in sibling plants that had inherited the transgene. The level of methylation increased in progeny of antisense-null plants but was still below normal. Remethylation of repeated sequences located at the centromere and at least some single copy sequences, including Ta3, occurred in first generation antisense-null plants (EJ Finnegan & T Kakutani, unpublished observations). Remethylation of endogenous sequences in these plants presumably occurred by de novo methylation.

Treatment of tobacco seed with 5-azaC results in extensive demethylation of HRS60 subtelomeric repeat DNA. Hypomethylated, HRS60 repetitive DNA was transmitted to progeny arising from self-pollination or outcrossing (140). In contrast, when tobacco suspension cells were subjected to a brief 5-azaC treatment, remethylation of the majority of DNA sequences occurred during several months' culture in the absence of the drug (1).

Thus, the capacity for de novo methylation in plants may be limited, particularly when global methylation patterns have been substantially altered.

ROLE OF DNA METHYLATION IN PLANTS

More than twenty years ago, Riggs (118) and Holliday & Pugh (57) proposed that cytosine methylation influences gene expression, a hypothesis based on the frequency of methylcytosine and on the proposed transmission of methylation patterns through cycles of DNA replication. It has since been shown that patterns of methylation are clonally inherited (146), that DNA methylation does repress transcription, and that changes in DNA methylation can be correlated with changes in gene expression in a tissue-specific or developmentally regulated manner (reviewed in 64). These data are correlative, and it is still not clear whether DNA methylation plays a primary role in regulating gene expression during development.

Methylation Changes During Development

If DNA methylation is important for tissue-specific or developmentally regulated gene expression, then there must be a mechanism to reset methylation patterns between generations. In mammals, global demethylation early in embryogenesis is followed by remethylation around the time of implantation (reviewed in 116). Germ cells are set aside early in development and are therefore not affected by organ-specific changes in methylation. Plants probably do not undergo global demethylation followed by remethylation during embryo development (46, 67) because *ddm1* mutant or methyltransferase antisense Arabidopsis plants that have a substantial reduction in DNA methylation do not restore normal levels of methylation in progeny that have lost the mutation or antisense transgene by outcrossing. Because the germ line is not set aside early in plant development, pro-gametic cells inherit methylation changes that accumulate in the vegetative meristem during development.

How are methylation patterns erased and reset in plants? The activity of maize transposable elements, *Ac* and *Spm*, differed when inherited from male or female gamete (42, 125), implying that methylation can change during gametogenesis. *Spm* and *Mu* elements are more heavily methylated in leaves at the top of the plant than in the first emerging leaves, showing that DNA methylation of transposable elements increases during development (6, 12, 86, 87).

Methylation levels of DNA from young seedlings were approximately 20% lower than in mature leaves of both tomato and Arabidopsis (92; EJ Finnegan, unpublished observation). In DNA from young Arabidopsis seedlings, the centromeric repeat was undermethylated compared to DNA of mature leaves (EJ Finnegan, unpublished observation), but it is not known whether methylation

of single-copy DNA also increases during development. Demethylation of these repetitive sequences probably occurred during gametogenesis or early in seed development because the centromeric repeat was also undermethylated in DNA from Arabidopsis seeds (EJ Finnegan, unpublished observation).

The level of DNA methylation was higher in tomato and Arabidopsis seeds than in mature leaves (92; EJ Finnegan, unpublished observation); this may be artefactual because the ratio of nuclear (methylated) to plastid (hypomethylated) genomes is probably higher in seed than leaf. If levels of methylation are increased in seed, then this may reflect increased methylation of single-copy sequences. Hypermethylation of single-copy DNA during embryogenesis, followed by demethylation of specific sequences during germination, would provide a mechanism for resetting methylation patterns in plants.

The data from Arabidopsis and maize indicate that methylation of transposable elements and other repeated sequences increases during development, perhaps to ensure that these sequences are packaged into heterochromatin before gamete formation. This could reduce meiotic recombination between repeated sequences (8, 13, 28, 33, 58, 134) at different chromosomal locations, which has the potential to cause major rearrangements leading to loss of gamete viability. Rearrangements resulting from recombination between nonallelic transposons is more common in yeast and Drosophila (74, 124), which lack DNA methylation, than in mammals (56) and plants (145).

A Role for DNA Methylation in Plant Development

Studies of the maize transposable elements, *Ac* and *Spm*, cycling between active and inactive states suggested that DNA methylation plays an important role in regulating the activity of these elements (7, 41, 126). The first indication that DNA methylation may regulate plant development came from work on the molecular basis of vernalization, that is, the promotion of flowering following prolonged exposure to low temperatures.

The vernalization signal is perceived by mitotically active cells that will form the inflorescence meristem; it is inherited through mitotic cell divisions but is not transmitted through meiosis (reviewed in 37). On the basis of these properties, Burn et al (29) proposed that vernalization is mediated by demethylation in the promoter of gene(s) whose expression is critical for the initiation of flowering and that subsequent expression of these genes triggers early flowering. Treatment with 5-azaC caused early flowering, mimicking the vernalization response and supporting the involvement of DNA methylation (27, 29). The early flowering response to chemical demethylation was specific to the vernalization-dependent flowering pathway because it was restricted to plants that respond to vernalization; there was no promotion of flowering in plants that are insensitive to vernalization (Figure 3) (27, 29, 37). Demethylation caused by a methyltransferase antisense construct also promoted flowering in the absence

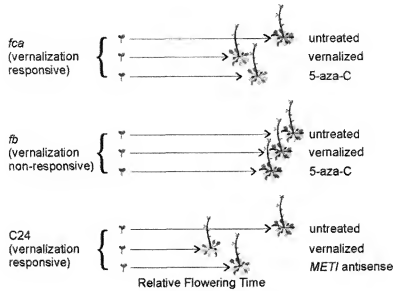


Figure 3 Changes in flowering time in response to vernalization; treatment with the demethylating agent, 5-azaC; or demethylation caused by a *METI* antisense construct. The length of the *line* separating the young seedling and flowering plant represents the flowering time in response to the treatment indicated, relative to the flowering time for the untreated control, which is taken as 100%. The late flowering mutants *fca* and *fb* are in ecotype L*er* (data adapted from 29), and the *METI* antisense is in ecotype C24 (46; RK Genger, unpublished observation).

of a cold treatment, again suggesting that decreased DNA methylation is sufficient to cause early flowering (Figure 3) (37; RK Genger & EJ Finnegan, unpublished observations).

One prediction from this hypothesis is that exposure to low temperatures will decrease methylation, perhaps by uncoupling replication and maintenance methylation. DNA methylation of cultured tobacco cells decreased after incubation for 1 week at 8°C (29). Similarly, a 4- or 8-week cold treatment of germinating Arabidopsis seeds caused a transient, 15% decrease in DNA methylation compared with control plants harvested at the same stage of development (EJ Finnegan, unpublished observation).

The cloning of genes required for the vernalization response will help elucidate any role of methylation.

Loss of DNA Methylation Affects Plant Development

DNA methylation is essential for normal plant development. Arabidopsis with reduced levels of DNA methylation display a range of abnormalities including loss of apical dominance, reduced stature, altered leaf size and shape, reduced

root length, homeotic transformation of floral organs, and reduced fertility (46, 67, 119). A subset of these morphological abnormalities was observed in independent plant lines in which methylation had been reduced either by mutagenesis or by introduction of a methyltransferase antisense gene (46, 67).

Decreased DNA methylation also altered flowering time in independent *METI* antisense and *ddm1* mutant plants; the effect depended on growth conditions and on the response of the wild-type progenitor to vernalization (65, 67, 119; RK Genger & K Kovac, unpublished observations). Other heterochronic changes in development occurred; some antisense plants formed aerial rosettes and produced more cauline leaves and secondary inflorescences on the primary bolt stem (119). Plants that had lost the antisense or *ddm1* mutation by segregation or outcrossing inherited both phenotypic abnormalities and reduced levels of DNA methylation, implicating demethylation as the cause of abnormal development (46, 66, 119).

Plants with the lowest methylation levels were most severely affected, and the abnormal phenotype became more severe in successive generations of progeny from self-pollinated plants (46). Although originally reported as having no phenotype, homozygous *ddm1* mutants developed phenotypic abnormalities after a number of generations of selfing; this correlated with demethylation of unique sequences (67). Arabidopsis plants in which methylation had been reduced by at least 70% became infertile after four to five generations of selfing (46). A comparable reduction in methylation is embryo lethal for mammals (81).

Melandrium album is a dioecious plant in which sex determination of the heterogametic male is based on the presence of a Y chromosome that plays a role in both male-determining and female-suppressing functions. Treatment with 5-azaC resulted in hermaphroditism in about 21% of male plants but did not affect sex determination of females (62). The karyotypically normal androhermaphrodites were sexual mosaics with both male and hermaphrodite flowers that showed differing degrees of gynoecium development and seed set. Although inheritance was incomplete, the bisexual trait was transmitted through two generations, but only when the androhermaphrodite plants were used as pollen donors. Janousek et al (62) proposed that female sex suppression in males depends on methylation of specific sequences either on the Y chromosome or on the autosomes. If an autosomal sequence is involved, then uniparental transmission suggests that this sequence must be imprinted following passage through the female gamete.

Imprinted Genes Are Important in Endosperm Development

The maternal and paternal genomes are not functionally equivalent in endosperm development (reviewed in 55, 69), a situation that is similar to genomic imprinting in mammals where the two parental genomes differ. The

endosperm of many angiosperms is triploid, having two copies of the maternal genome and a single copy of the paternal genome (82). When this balance is perturbed, development is impaired, often leading to failure of seed maturation. Using translocations, it has been shown that some chromosomal arms must be derived from the male parent for normal endosperm development to occur (55, 69); these arms may carry imprinted genes that are essential for endosperm development. Nonessential genes, *r* and *dzr1*, are also subject to imprinting (30, 68); differential methylation, which correlated with differential expression, of maternal and paternal copies of alleles at the *r* locus has been observed (M Alleman, unpublished observation), suggesting that, as in mammals (80), DNA methylation may also be important for genomic imprinting in plants.

Imprinting in plants differs from mammalian genomic imprinting in two aspects. First, even though differential activity of transposable elements has been observed after transmission through male and female gametes (42, 125), imprinting has so far been demonstrated only in endosperm, which does not contribute to the next generation (55, 69). Second, not all alleles at imprinted loci show differential expression and/or methylation (30, 68; M Alleman, personal communication).

DNA Methylation Modulates Endogenous Gene Expression

The Arabidopsis *PAI* genes encoding phosphoribosylanthranilate isomerase, an enzyme of the tryptophan pathway, are examples of endogenous genes whose expression is modulated by methylation. The ecotype Ws has four copies of the gene at three unlinked loci. All four genes are methylated in the homologous coding sequences, but sufficient enzyme is made to prevent tryptophan deficiency (10). In a spontaneous tryptophan mutant, the two linked genes *PAI1* and *PAI4*, present as a tail-to-tail inverted repeat, were deleted by recombination between flanking, direct-repeat sequences. The mutant phenotype was unstable and occasionally yielded revertant progeny or somatic sectors. Reversion of the mutant phenotype was associated with increased expression and hypomethylation of the two remaining unlinked genes, *PAI2* and *PAI3* (10). When the *ddm1* mutation was introduced into the *pai1-pai4* deletion, hypomethylation and reactivation of *PAI2* and *PAI3* genes occurred at high frequency (J Jeddeloh, personal communication). When the complex, methylated Ws *PAI1-PAI4* locus was combined with unmethylated *PAI2* and *PAI3* genes by crossing to another ecotype, the unmethylated copies became methylated (J Bender, personal communication), a process resembling *trans*-inactivation of 9 transgene (91).

When independent homozygous *ddm1* mutant lines were established by five generations of selfing, their progeny displayed overlapping sets of phenotypic

abnormalities, suggesting that a limited number of genes was affected (66). Five phenotypic traits were inherited, independent of the *ddm1* mutation, in F2 outcross progeny. These epimutations have been mapped to loci unlinked to *DDM1*, and attempts to clone the genes responsible are under way (65, 66).

Reduction in DNA methylation in *METI* antisense plants is associated with aberrant expression of several floral regulatory genes. Ectopic expression of *APETALA3* and *AGAMOUS* (*AG*), which are normally expressed in floral tissue, was observed in leaves (46); expression was low and was probably not the cause of the floral abnormalities. Another gene, *SUPERMAN* (*SUP*), showed wild-type expression in the ovule but was ectopically expressed in the carpel wall (H Sakai & EJ Finnegan, unpublished observation). In wild-type flowers, *SUP* is also expressed in the floral bud (121); in situ hybridization showed that the genes, *SUP* and *AG*, were not transcribed in the developing floral buds of antisense plants that had *sup* or *sup ag* mutant flowers, respectively (EJ Finnegan & H Sakai, unpublished observation). Methylation of the *SUP* gene was examined in wild-type and in *METI* antisense plants with *sup* flowers. Restriction enzyme analyses showed that sites some distance from the gene were methylated in wild-type but not in antisense plants (N Kishimoto & EJ Finnegan, unpublished observation). Genomic sequencing of the *SUP* gene in wild-type plants showed that there was no cytosine methylation in the coding sequence or within 1 kb upstream of the transcription start. Unexpectedly, the corresponding region was densely methylated in the *METI* antisense plants (61; N Kishimoto & EJ Finnegan, unpublished observation). Hypermethylation of cytosines located in both symmetric and nonsymmetric sequences was associated with repression of *SUP* transcription in the developing flower bud and with a *sup*-like phenotype. Repression of *AG* was also associated with hypermethylation of this gene in *METI* antisense plants (S Jacobsen, personal communication). It is likely that methyltransferases other than METI methylate these sites; transcripts of *METII* were present in *METI* antisense plants (RK Genger, unpublished observation).

Hypermethylation at the *SUP* gene was also observed in seven independent lines that showed a weak, unstable *sup* phenotype, termed *clark kent* (*clk*). These *clk* mutants were isolated from plants that had undergone EMS, diepoxybutane, fast neutron, X irradiation, or insertional mutagenesis (61). The initiating event for hypermethylation of *SUP* in these plants is not known.

There are other examples of mutations in Arabidopsis that are associated with local changes in DNA methylation. Two independent mutations at the *fwa* locus, which confers late flowering, are associated with hypomethylation of at least 5 Mb, spanning *FWA*, as determined by methylation RFLPs (W Soppe & M Koornneef, personal communication). A semidominant late flowering mutant that was identified in *ddm1* mutants and that can be segregated from *ddm1* maps at *FWA* (65); perhaps the late flowering phenotype in this line is also associated

with hypomethylation. Mutagenesis may result in perturbation of methylation patterns at unlinked sites because the *clk1* allele arose in the same mutagenesis as the *fwa1* mutant.

The observation that de novo methylation can be stimulated by global demethylation and by mutagenic agents suggests that plants use DNA methylation as a defense against factors that have the potential to perturb normal genome organization. The local hypermethylation observed at *SUP* and *AG* resembles that seen in silenced transgenes and inactive transposable elements. It is possible that changes in chromatin structure caused by demethylation, mutagenesis, or insertion of mobile elements or transgenes stimulates dense methylation. It is likely that genes involved in flowering are no more susceptible to changes in methylation than other genes, but are more easily scored.

DNA Methylation Is Associated with Transgene Silencing

Transgenes are a tool both to investigate gene function and to improve plants of agronomic importance by improving characters. One limitation to plant improvement is that transgenes are frequently inactivated, by at least two independent mechanisms (reviewed in 45, 48, 88, 131). In transcriptional silencing, RNA production from the introduced gene is repressed. In posttranscriptional silencing, transcripts do not accumulate in the cytoplasm even though transcription rates are comparable with or higher than those in cells where transcripts do accumulate (99).

Transcriptional silencing is associated with transgene methylation, particularly in the promoter (84). Methylated transgenes are packaged into condensed chromatin, as determined by reduced accessibility to nucleases (94, 149; R van Blockland & P Meyer, personal communication). Such silenced transgenes can be reactivated by treatment with 5-azaC (143) or by crossing into a *ddm1* mutant background (O Mittelsten Scheid, personal communication); in each case, transgene methylation decreased, suggesting that methylation plays an integral part in the maintenance of silencing. However, introduction of a *METI* antisense construct did not reactivate the same 35S*Hyg* transgene reactivated in *ddm1* homozygotes, even though some demethylation occurred (O Mittelsten Scheid, personal communication). The *METI* antisense does not remove methylation of cytosine in all sequence contexts.

DNA structure may be one factor that identifies foreign DNA as a target for methylation, as silencing occurs more frequently when multiple copies of the transgene are inserted (4, 84), perhaps because of pairing of transgenes at the same or different loci (88). Inverted transgene repeats may form a cruciform structure, a structure that is recognized and efficiently modified by mammalian methyltransferases (15, 129). Other proposed mechanisms for targeting foreign DNA include lack of homology between sequences flanking paired

transgenes inserted at different loci (ER Signer, cited in 20), RNA-mediated de novo methylation (142), or differences in base composition between a transgene and flanking DNA (95). For example, the maize *A1* gene, which encodes dihydroflavonol-4-reductase (DFR), is frequently silenced in transgenic Petunia; silencing was associated with methylation of multiple copy inserts (40, 91a). When the Gerbera homologue (*gdfr*), which has a C+G content more compatible with the Petunia genome, was used in place of the *A1* gene, multiple copy insertions of the transgene remained unmethylated and flowers were intensely pigmented (40). Most reports of transcriptional silencing involve transgenes from heterologous sources, suggesting that DNA heterogeneity caused by insertion of foreign DNA may be a common theme in transgene inactivation.

The frequency of inactivation of a single copy of the chimeric 35S*A1* gene in transgenic Petunia was increased by a prolonged period of elevated temperatures and high light intensity. There was a parallel increase in methylation of the transgene (96). Methylation may be increased by heat and/or high light intensity, contrasting with demethylation, which appears to accompany vernalization.

Posttranscriptional silencing, which affects both transgenes and homologous endogenous genes, is also associated with transgene methylation, but within the coding sequence rather than the promoter (59, 128). It is not clear whether methylation is required for posttranscriptional silencing. Expression of a transgene that has homology to sequences present in the genome of an invading RNA virus can lead to degradation of the viral RNA and resistance to infection (83).

It is likely that both forms of transgene silencing reflect normal cellular defenses against invading or mobile DNAs (45). Posttranscriptional silencing is functionally equivalent to natural processes that allow tobacco plants to recover from, and develop immunity to, infection by nepoviruses (115). Transcriptional silencing is similar to inactivation of transposable elements, retroelements, and T-DNA. *Trans*-inactivation of an incoming transgene by an inactive, resident transgene (91), resembles paramutation (90), which has been defined as a heritable change in one allele (the paramutable allele) induced by a second, paramutagenic allele (26). Changes in DNA methylation are detected by restriction enzymes associated with paramutation at the maize *r* locus (M Alleman & J Kermicle, cited in 107). To date, no changes in methylation have been detected using restriction enzymes after paramutation of the *b* locus of maize (106, 107).

IS DNA METHYLATION A PRIMARY OR SECONDARY MECHANISM FOR REGULATING GENE EXPRESSION?

There are many examples in plants of genes that are both transcriptionally inactive and methylated within the promoter and/or coding sequences. It is not

clear, however, whether DNA methylation is the primary cause of inactivation or a secondary consequence of some other process that has resulted in transcriptional inactivation.

It can be difficult to determine cause and effect, but the *Pl-Blotched* (*Pl-Bh*) allele of the maize purple plant (*Pl*) gene, encoding a transcription factor that regulates anthocyanin production, provides an example where changes in methylation are secondary to alterations in chromatin structure (O Hoekenga & K Cone, personal communication). The *Pl-Bh* allele causes variegated pigmentation throughout the plant, which ranges from heavy blotching at the base to sparse blotching at the top. In husk tissue, decreased pigmentation was associated with hypermethylation of *Pl-Bh* compared with *Pl-Rhoades*, an allele that conditions uniform intense pigmentation (34). Hypermethylation correlated with decreased sensitivity to DNase I and with reduced gene expression of *Pl-Bh*. *Pl-Bh* was also resistant to DNase I in juvenile tissue where methylation RFLP analyses showed that the gene was hypomethylated (O Hoekenga & K Cone, personal communication). In this case, the primary event appears to be compaction of chromatin around *Pl-Bh* followed by hypermethylation. Because the two alleles show only 10 nucleotide changes over 5.5 kb, including the coding region and flanking sequences, it is likely that differences in expression relate to chromatin structure. The patchy expression of *Pl-Bh* throughout the plant may result from chromatin condensation, with the density of blotches decreasing as this structure is stabilized by methylation.

Organisms lacking methylated DNA, such as Drosophila, regulate gene expression by establishing chromatin structures that are stably inherited through mitosis and that are compatible with either transcriptionally active or repressed states. Polycomb-group (Pc-G) proteins are found in chromatin associated with stably repressed genes and Trithorax-group (Trx-G) proteins with transcribed genes (reviewed in 105, 112). The protein encoded by the Arabidopsis *CURLY LEAF* (*CLF*) gene has sequence and functional homology to *E(z)*, a Pc-G protein of Drosophila (51). Long-term repression of the *AG* gene is dependent on CURLY LEAF; in *clf* mutants, *AG* is expressed ectopically in leaves and in flower buds at a late stage in development. If DNA methylation stabilizes chromatin structure in plants (46), then perturbation of DNA methylation could affect chromatin structure around *AG*. The observation that *AG* is expressed ectopically in leaves of methyltransferase antisense plants is consistent with this prediction (46).

METIII, a putative methyltransferase containing a chromodomain, provides a link between DNA methylation and chromatin structure, as chromodomain proteins Hp1 and Pc from Drosophila are associated with condensed chromatin (105). If METIII is associated with compacted chromatin via the chromodomain this could direct methylation of the underlying DNA. Alternatively, compaction of DNA could arise by association of other chromodomain proteins

with METIII during methylation. Dnmt1, the mouse methyltransferase, has homology to a human homologue of the trithorax protein (Trx), again suggesting a link between DNA methylation and chromatin (21). Dnmt1-like plant methyltransferases lack homology to Trx.

CONCLUSIONS

In both prokaryotes and lower eukaryotes, DNA methylation provides a defense against invading or mobile DNA (52, 102, 127). Duplicated sequences in *Ascobolus immersus* and *Neurospora crassa* are methylated and inactivated just prior to cells entering the sexual phase of the life cycle (52, 127). Methylation prevents recombination between nonallelic repeat sequences and the spread of mobile elements in these fungi (53, 120). Mobile elements and other repeated sequences are methylated in plant genomes, supporting the idea that DNA methylation also plays an important role in genome defense in plants.

Reduction of methylation in Arabidopsis demonstrated that methylation is essential for normal development. In methyltransferase antisense plants with reduced levels of DNA methylation, genes involved in flower development showed both low-level ectopic expression and transcriptional repression associated with hypermethylation (46, 61). Flowering time was altered, consistent with DNA methylation having a role in vernalization. The dysregulation of gene expression in these plants implies that even if methylation of DNA does not play a primary role in regulating gene expression it provides the appropriate genomic context essential for programmed development.

DNA methylation has dual roles in defense and gene regulation. How did this arise? Plant genomes are punctuated with methylated retroelements and their remnants. Many of these elements have inserted into intergenic spacers, and some are located within promoter regions contributing sequences important for promoter function (138). Did an ancient role in genome defense evolve into one of gene regulation because methylated retroelements reside in promoters? Did a primary role in regulating gene expression in complex genomes expand to include genome defense? Or was methylation independently recruited to control gene expression and defend against invading DNA in complex genomes?

While it is unlikely that we will be able to answer these questions from studies of DNA methylation, we may gain insights into what enables a plant to activate defense mechanisms to silence invading DNA. Gene inactivation by hypermethylation in the *MET1* antisense plants suggests that a defense mechanism has been activated against endogenous genes. An understanding of the factors that regulate this process may provide insights into what makes an endogenous gene appear as foreign DNA. This in turn may assist in the generation of transgenic plants that stably express transgenes.

ACKNOWLEDGMENTS

The authors thank the following people for allowing inclusion of results prior to publication: R Adams, M Alleman, J Bender, T Bestor, L Comai, K Cone, T Elmayan, S Henikoff, O Hoekenga, S Jacobsen, J Jeddeloh, T Kakutani, N Kishimoto, K Kovac, M Koornneef, M Matzke, A Matzke, P Meyer, O Mittelsten Scheid, J Paszkowski, S Pradhan, E Richards, H Sakai, W Soppe, R van Blockland, H Vaucheret, and J Yoder; and L Thorpe for assistance in preparation of the manuscript.

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